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Rhox: A New Homeobox Gene Cluster

James A. MacLean, II,[1] Mingang A. Chen,[1] Chad M. Wayne,[1] Shirley R. Bruce,[1] Manjeet Rao,[1] Marvin L. Meistrich,[2] Carol Macleod,[3] and Miles F. Wilkinson[1,*]

[1]Department of Immunology

[2]Department of Experimental Radiation Oncology
M.D. Anderson Cancer Center
University of Texas
Houston, Texas 77030

[3]Cancer Center
Department of Medicine
School of Medicine
University of California at San Diego
La Jolla, California 92093

Summary

Homeobox genes encode transcription factors notable for their ability to regulate embryogenesis. Here, we report the discovery of a cluster of 12 related homeobox genes on the X chromosome expressed in male and female reproductive tissues in adult mice. These *r*eproductive *ho*meobox on the *X* chromosome (*Rhox*) genes are expressed in a cell type-specific manner; several are hormonally regulated, and their expression pattern during postnatal testis development corresponds to their chromosomal position. Most of the *Rhox* genes are expressed in Sertoli cells, the nurse cells in direct contact with developing male germ cells, suggesting that they regulate the expression of somatic-cell gene products critical for germ cell development. In support of this, targeted disruption of *Rhox5* increased male germ cell apoptosis and reduced sperm production, sperm motility, and fertility. Identification of this family of homeobox genes provides an opportunity to study colinear gene regulation and the transcriptional control of reproduction.

Introduction

Homeobox genes encode transcription factors containing a 60 amino acid DNA binding motif called a homeodomain. Homeodomain transcription factors regulate many embryonic developmental programs, including axis formation, limb development, and organogenesis (Weatherbee et al., 1998). Some homeodomain transcription factors are also expressed in adult tissues such as liver, kidney, and intestine, where they are thought to govern regenerative differentiation of cells (Cillo et al., 2001).

The best-known homeobox gene subclass is the *Hox* family. The *Hox* genes are present in large clusters, apparently to maintain the temporal and spatial colinear regulation of their expression so that they can properly regulate the development of their target tissues. The initial discovery of a *Hox* gene cluster was reported nearly three decades ago in *Drosophila melanogaster* (Lewis, 1978). In mammals, the *Hox* genes appear to have arisen through duplication of individual genes or multigene units, which subsequently evolved specialized functions or were lost through selection (Martinez and Amemiya, 2002).

The clustering of *Hox* genes allows unique opportunities for organized gene regulation germane to their role in body-plan formation. Strikingly, many *Hox* genes display a colinear pattern of expression determined by their position in the cluster. At least three types of colinearity have been described (Duboule and Morata, 1994). In spatial colinearity, the position of a gene in a cluster correlates with its expression domain; e.g., 5′ and 3′ *Hox* genes are typically expressed in the posterior and anterior portions of developing embryos, respectively. Genes displaying temporal colinearity are activated at progressively later times during development, such that genes at one end of the cluster are turned on first and genes at the opposite end of the cluster are turned on last. Quantitative colinearity refers to a correlation between gene position and expression level, such that the first gene within a cluster displays the maximum level of mRNA expression and downstream genes exhibit progressively lower expression. While the colinear expression pattern of *Hox* genes has long been known, only recently have some regulatory regions (e.g., global enhancers) been defined that specify it (Spitz et al., 2003).

Here we report the discovery of a new homeobox gene cluster on the mouse X chromosome expressed selectively in male and female reproductive tissues. These *r*eproductive *ho*meobox *X*-linked (*Rhox*) genes are expressed in a cell type-specific manner; some exhibit hormone-dependent expression, and all exhibit a colinear expression pattern that precisely corresponds with their position within subclusters on the X chromosome. Targeted disruption of one of these genes reduces spermatozoa output from the testis, decreases the proportion of motile spermatozoa in the epididymis, and causes subfertility. We suggest that the Rhox proteins are a new family of transcription factors that regulate diverse developmental events in the male and female reproductive tracts.

Results

Identification of the *Rhox* Homeobox Gene Cluster on the X Chromosome

We previously mapped the *Pem* gene to the *Hprt* region of the mouse X chromosome (Sutton and Wilkinson, 1997b). Our subsequent analysis of the mouse genome database confirmed this mapping and allowed us to more precisely localize *Pem* to the A2 region (Figure 1A). Further scrutiny of the mouse genome revealed that *Pem* is not alone on the X chromosome but rather is part of a cluster of 12 related homeobox genes within an ~0.7 Mb stretch of the A2 region (Figure 1A). We

*Correspondence: mwilkins@mdanderson.org

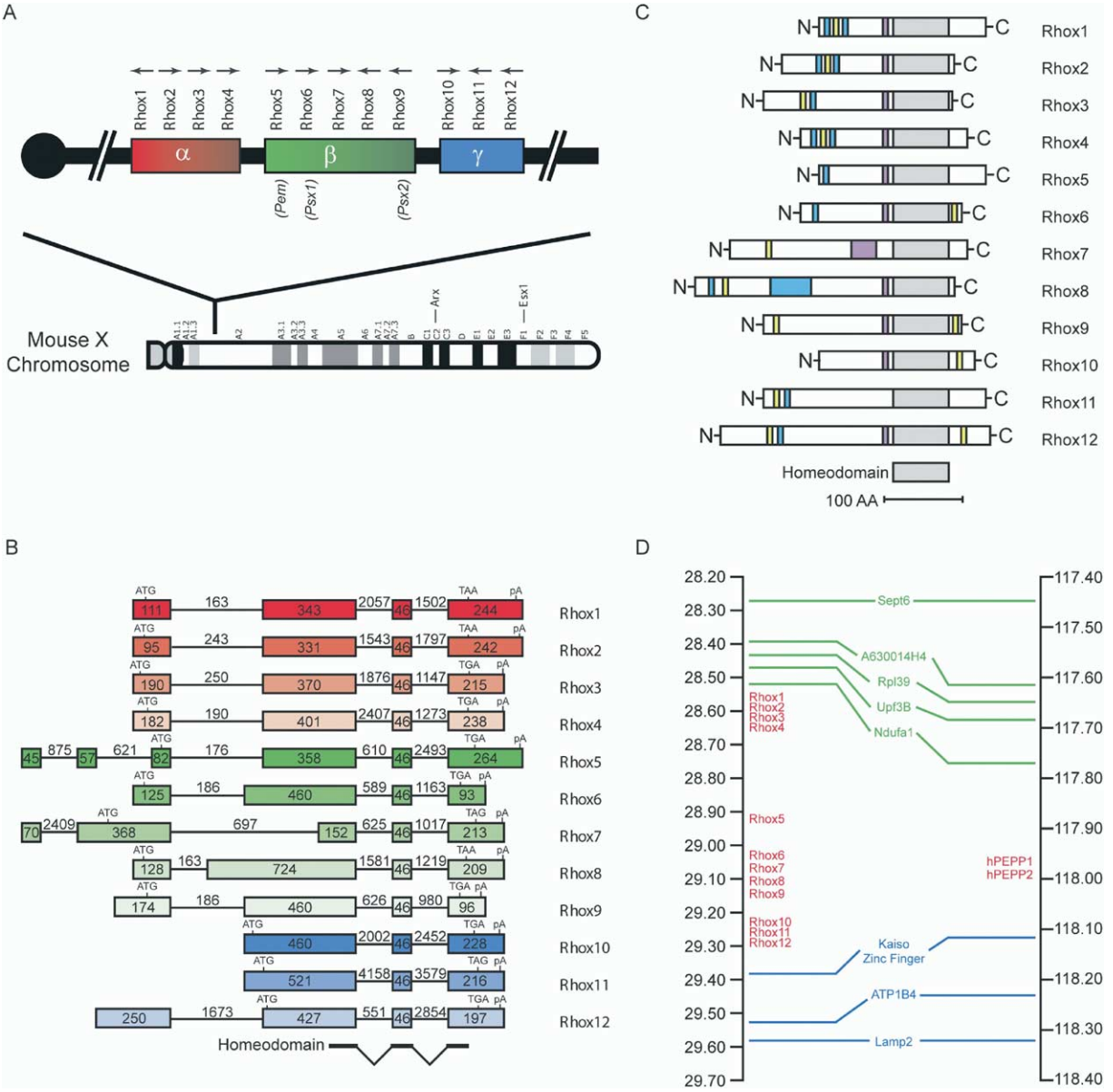

Figure 1. The *Rhox* Homeobox Gene Cluster

(A) All 12 *Rhox* genes are contained within an ~0.7 Mb segment of the A2 region. The genes are further divided into three subclusters: α, β, and γ based on proximity, expression patterns, and sequence identity.

(B) The exon-intron structure of individual *Rhox* genes. The putative start codons (ATG) and polyadenylation signals (pA) are indicated.

(C) The *Rhox* genes are predicted to produce proteins of similar length, each with a single homeodomain near the C terminus (gray box). Clusters of residues with similar biochemical properties are indicated by colored bands: positively charged (purple), hydrophobic (yellow), and negatively charged (blue).

(D) The position of the mouse *Rhox* gene cluster and the human *RHOX* (*hPEPP1* and *hPEPP2*) genes relative to neighboring genes on the mouse and human X chromosomes, respectively. Numbers indicate the map distances from the centromere according to the Ensembl database (build 32).

named the genes in this cluster the *Rhox* genes because of their selective expression in reproductive tissues, as we describe later. The genes were designated by number according to their order on the X chromosome; the gene closest to the centromere was named *Rhox1*; the most distant was named *Rhox12*. According to this nomenclature, *Pem* is *Rhox5*, and the other genes that have been previously identified, *Psx1* and *Gpbox* (*Psx2*) (Chun et al., 1999; Takasaki et al., 2000) are *Rhox6* and *9*, respectively. Interestingly, the *Rhox4* gene is 97% identical over its entire length with the genomic sequence reported in Ensembl for *Ehox*, a previously characterized homeobox gene (Jackson et al., 2002). *Rhox8* was recently described elsewhere as the

Tox (testis and ovary homeobox) gene (Kang et al. 2004).

The deduced amino acid sequence of each Rhox protein revealed that they contain related homeodomains. Each has the hydrophobic residues critical for homeodomain packing (Duboule, 1994), including the highly conserved W and F residues found in virtually all known homeodomains at positions 48 and 49, respectively (Figure 2A). Interestingly, the amino acids predicted to dictate base-specific DNA binding (marked in bold and with a "b" in Figure 2A) are, in general, different from those in most known homeodomain proteins, suggesting that Rhox proteins have novel DNA targets (see Discussion).

Figure 1B shows the exon-intron structure of all 12 *Rhox* genes. We used the mouse genome database and the exon predictor program Genescan to guide us in deducing the exon-intron structures. While useful, these approaches often misidentified splice sites, most commonly in 5′ exons. The correct exon-intron junctions were determined by visual inspection of potential splice sites, examination of expressed sequence tags corresponding to *Rhox* genes, trial-and-error assessment of suspected exons by RT-PCR analysis, and sequence analysis of these RT-PCR products.

Several lines of evidence indicated that the *Rhox* gene cluster represents a new homeobox subfamily: first, all 12 *Rhox* genes have two introns at identical positions in the homedomain region: one intron within codon 31 and the other intron between codons 46 and 47 (Figure 1B). Two other X-linked homeobox genes, *Arx* and *Esx1*, which are distant from the *Rhox* gene cluster on the X chromosome (Figure 1A), share the placement of these introns. This contrasts with most other homeobox genes, which either have no intron in the homeodomain region or contain a single intron at various positions in the homeodomain (Duboule, 1994). Second, the homeodomain is located at a conserved position in all 12 Rhox proteins (Figure 1C). Third, the length of most of the Rhox proteins is very similar, differing at most by 10%. The only exception is Rhox8, which includes a Glu repeat domain of about 110 residues encoded in its large exon 2 (Figure 1B). Fourth, phylogenetic analysis, comparing Rhox homeodomains with all 166 known complete mouse homeodomain sequences deposited in the NIH Genome Research Institute database, indicated that the Rhox homeodomains are more related with each other than they are with other known homeobox gene families (Figure 2B). This also indicated that the *Rhox* cluster forms a new homeodomain subfamily most closely related to the *Paired*- and *Prd*-like families. Consistent with this, *Rhox* genes share with some *Paired*- and *Prd*-like gene family members an intron between homeodomain codons 46 and 47 (Duboule, 1994).

Within the *Rhox* gene family, phylogenetic analysis indicated that the 5′ *Rhox* genes (subcluster α) and 3′ *Rhox* genes (subcluster γ) (Figure 1A) tended to group tightly together as distinct branches (Figure 2B). For example, the subcluster α genes *Rhox10* and *11* are quite related, as they encode homeodomains with 65% sequence identity (Figure 2A). In contrast, the subcluster β genes displayed variable degrees of relatedness (Figures 2A and 2B). The most related are the *Rhox6* and *9* genes, which encode homeodomains that are 80% identical. In contrast, *Rhox7* is more related the subcluster α gene, *Rhox4*, than to other subcluster β members, based on homeodomain sequence.

Two human homeobox genes, *hPEPP1* and *hPEPP2* (*OTEX*) previously identified by us and others (Geserick et al., 2002; Wayne et al., 2002) are likely to be orthologous to one or more members of the *Rhox* gene cluster. We could not establish definitively whether the only human homeobox genes in the region syntenic to the *Rhox* gene cluster are *hPEPP1* and *hPEPP2*, but neither manual scanning of predicted genes nor BLAST searches of contigs overlapping this region of the human X chromosome have revealed any other human homeobox genes in this region.

Tissue-Specific Expression Patterns of the *Rhox* Genes

To determine the expression pattern of the *Rhox* genes, we employed real-time RT-PCR analysis using primers that were specific for each one. A panel of 18 adult tissues and placenta was screened. This analysis revealed that nine of twelve *Rhox* genes are expressed exclusively in reproductive tissues and placenta. Three *Rhox* genes (*Rhox4*, *7*, and *8*) are also expressed in one additional tissue (Figure 3; note that it depicts expression on a log scale).

Northern blot analysis validated the real-time RT-PCR analysis and provided the sizes of the *Rhox* transcripts (see Supplemental Figure S1 in the supplemental data available with this article online). In agreement with RT-PCR analysis, Northern blot analysis showed that *Rhox3*, *8*, and *11* are expressed at their highest levels in the testis, while *Rhox2*, *4*, *5*, *6*, *9*, *10*, and *12* are predominantly expressed in the placenta and *Rhox1* is most abundantly expressed in ovary. None of the *Rhox* transcripts were detectable in liver.

Rhox Genes Display a Temporal and Quantitative Colinear Pattern of Expression

The expression of the *Rhox* gene cluster in reproductive tissues suggested that its members are good candidates to govern gametogenesis and thereby to promote or regulate fertility. To narrow down which developmental events they might regulate during male gametogenesis, we analyzed their developmental expression pattern using real-time RT-PCR. We were intrigued to find that the expression pattern of each *Rhox* gene corresponds to its chromosomal position. In particular, we found that the *Rhox* genes segregate into three subclusters (α, β, and γ) that each display a progressive pattern of expression (Figure 4).

The genes in subcluster α display both temporal and quantitative colinearity, such that the timing and level of their peak expression during postnatal testis development corresponds to their position within the subcluster. *Rhox1*, the most 5′ gene (with respect to the centromere) in subcluster α is expressed first (between days 7 and 12 postpartum), and then expression rapidly falls off (Figure 4A). The next gene (*Rhox2*) is expressed at a later point of development, peaking around day 12 postpartum. *Rhox3* and *4* are expressed at progressively later points of development, peaking be-

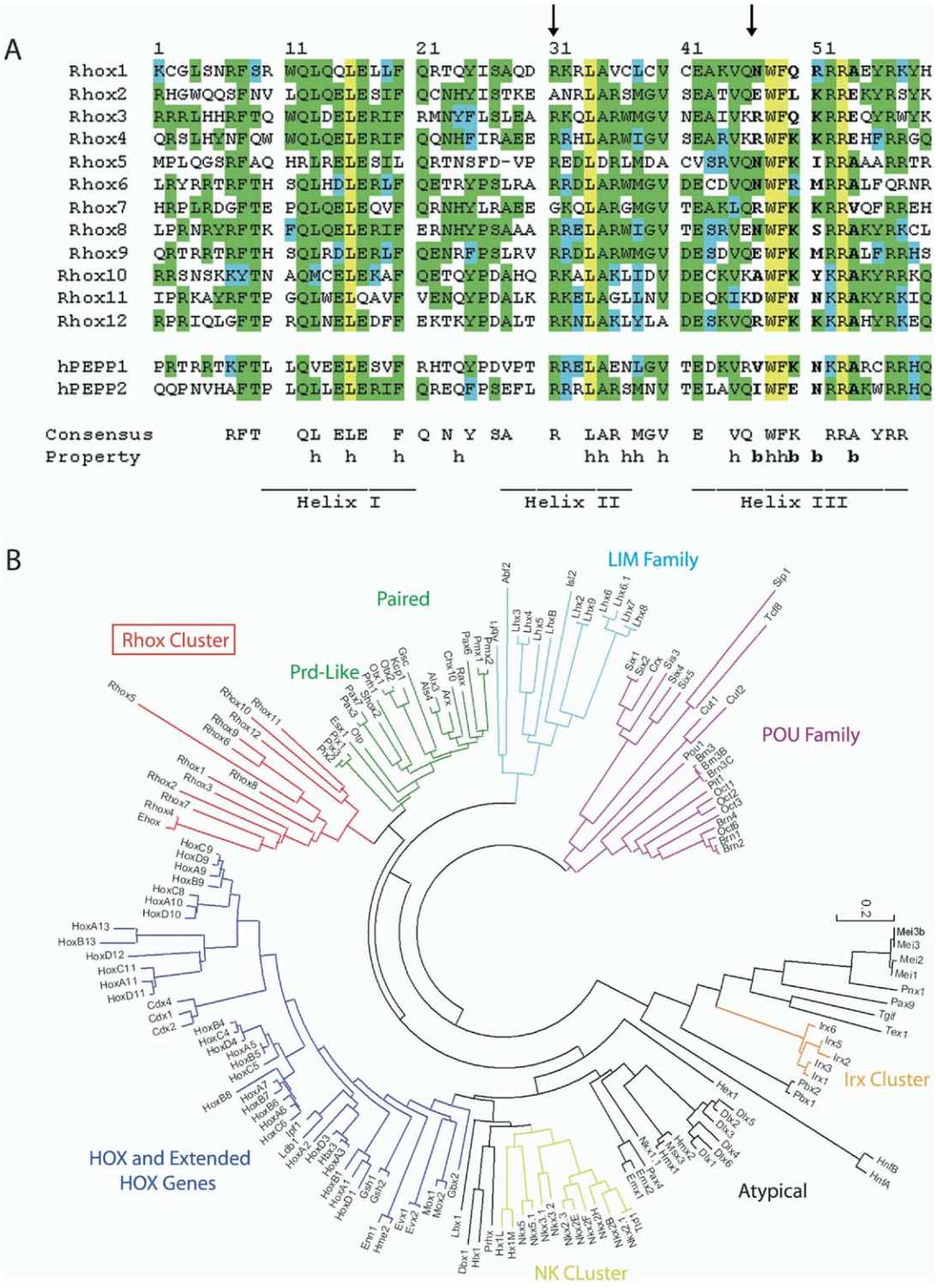

Figure 2. Phylogenetic Comparison of the Rhox Homeodomains

(A) Alignment of the predicted amino acid sequences of the *Rhox* homeodomains. Yellow-shaded residues are absolutely conserved; green-shaded residues are the most common ones at each position, and blue-shaded residues are similar amino acids to those conserved. The position of hydrophobic amino acids known to be essential for homeodomain packing are indicated with an "h." The residues involved in DNA base pair-specific contacts are indicated with a "b." The positions of the two introns that bisect all 12 Rhox homeodomains are indicated with arrows.

(B) Unrooted phylogenetic tree constructed by the neighbor joining method. Branch lengths represent the extent of divergence.

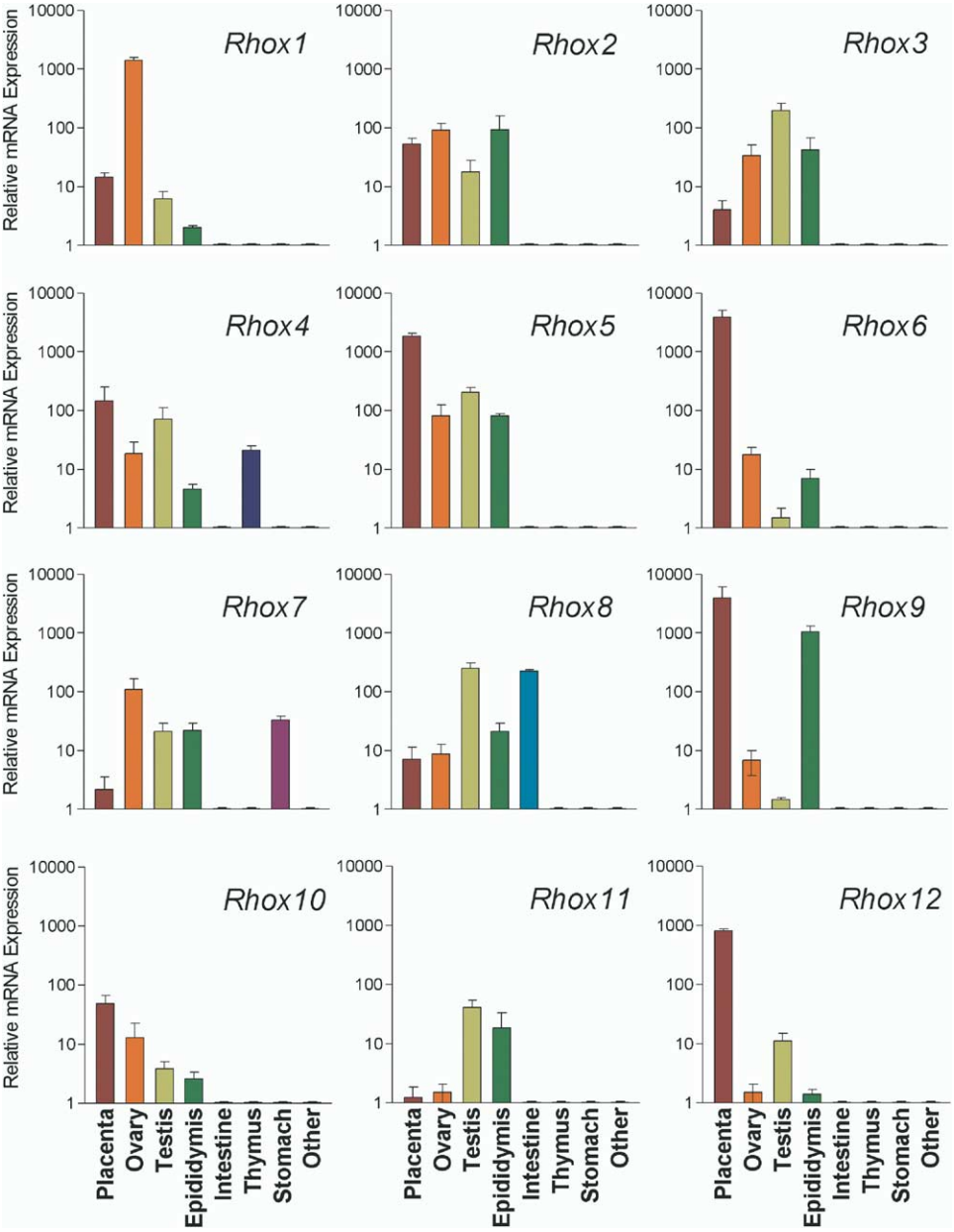

Figure 3. Preferential Expression of *Rhox* Genes in Reproductive Tissues

Real-time RT-PCR was used to quantitate the level of *Rhox* mRNA relative to L19 mRNA in total cellular RNA from the tissues shown. Columns represent the average fold increase ± standard error (SEM) of *Rhox* gene expression over background for at least two separate RT reactions assayed in duplicate (note that values are on a log scale). The only tissues shown are those that detectably expressed at least one *Rhox* gene. The tissues that did not have detectable expression were: brain, skin, heart, lung, spleen, tongue, kidney, liver, seminal vesicle, prostate, and vas deferens, which are grouped together under "other."

tween days 20 and 22 postpartum. *Rhox1* is expressed at a higher level during testis development than the other subcluster α genes. Each subsequent gene in subcluster α exhibits a stepwise decline in mRNA level such that *Rhox4* mRNA is barely expressed in the testis (~50-fold lower levels than that of *Rhox1*).

To validate the real-time RT-PCR analysis, we performed ribonuclease protection analysis. The results were nearly superimposable with those from real-time RT-PCR analysis (Figure 4A). We conclude that the temporal and quantitative expression pattern of these four *Rhox* genes, all of which are clustered together in a 56 kb region, correspond to their position within that cluster.

The genes in subcluster β do not exhibit temporal colinearity but they do display a quantitative colinear pattern of expression (Figure 4B). Peak expression levels progressively decline for the genes in this subcluster such that the most 3′ gene in the subcluster, *Rhox9*, is completely silent in the testis (judged by real-time RT-PCR analysis at all time points; data not

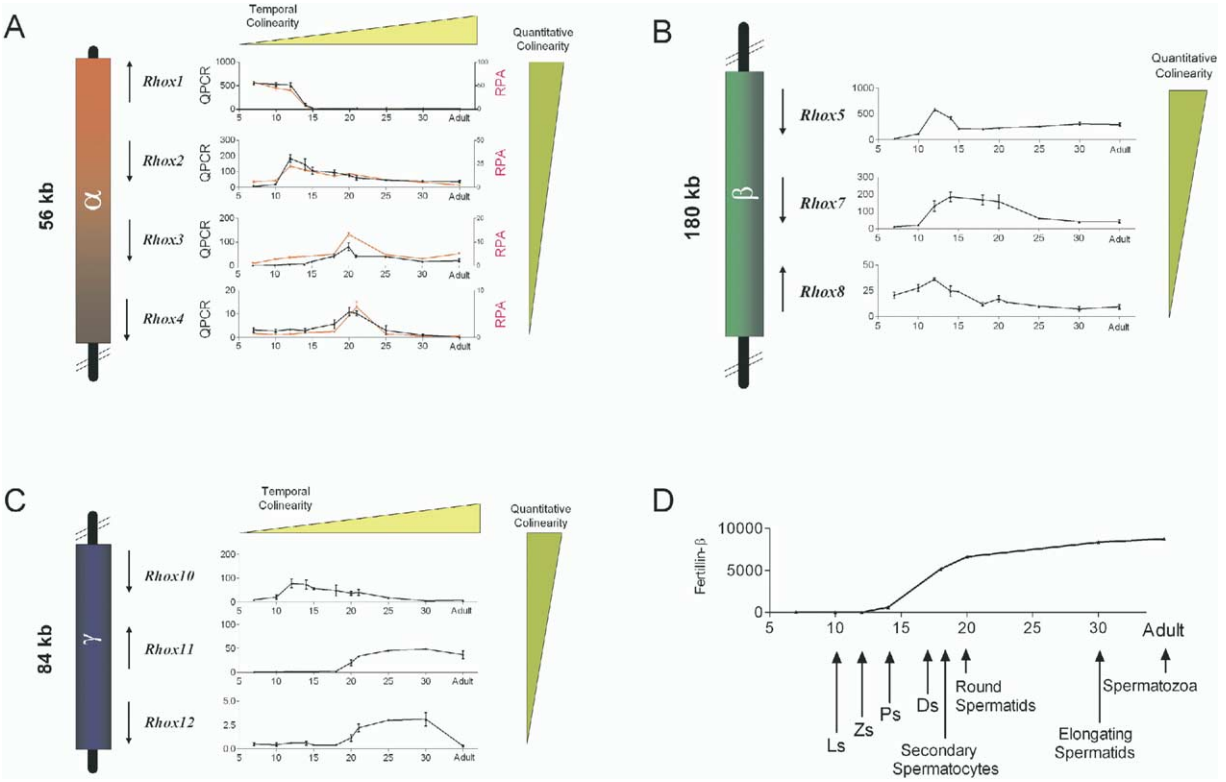

Figure 4. Developmentally Regulated Expression of the *Rhox* Gene Cluster

Rhox and fertillin-β gene expression (relative to L19 mRNA, as in Figure 3) assessed in total cellular RNA prepared from testes from mice of the ages shown (adult mice are 60+ days old; no breakpoint is indicated on the x axis scale).

(A) Real-time RT-PCR analysis (left y axis) and ribonuclease protection analysis (performed as described in Rao et al. [2003]) (right y axis) of *Rhox* subcluster α expression during testis development. These genes exhibit temporal and quantitative colinear regulation (indicated by the triangle).

(B) Real-time RT-PCR analysis of *Rhox* subcluster β.

(C) Real-time RT-PCR analysis of *Rhox* subcluster γ.

(D) The expression pattern of fertillin-β during postnatal testis development. The developmental time points marking the initial appearance of key cell populations during the first wave of spermatogenesis are indicated. Primary spermatocytes are abbreviated as follows: leptotene (Ls), zygotene (Zs), pachytene (Ps), and diplotene (Ds).

shown). Another gene in this cluster, *Rhox6*, is also not expressed in the testis.

Like subcluster α, subcluster γ exhibits both temporal and quantitative colinearity (Figure 4C). The first gene, *Rhox10*, is expressed early, exhibiting a temporal expression pattern similar to that of the first gene in subcluster β. The next genes, *Rhox11* and *12*, initiate expression once *Rhox10* mRNA levels begin to wane. Peak expression levels of the three genes in subcluster γ matched their positions: the most 5′ gene (*Rhox10*) is expressed at the highest level, the middle gene (*Rhox11*) is expressed at a moderate level, and the most 3′ gene (*Rhox12*) is expressed at the lowest level.

Rhox Genes Exhibit Cell Type-Specific Expression

To identify the cell types in the testis that express the *Rhox* genes, we purified testicular cell fractions enriched for Sertoli and interstitial (e.g., Leydig) cells. The majority of the Rhox genes are expressed primarily in Sertoli cells. The lone exception was Rhox4 which was predominantly Leydig cell in origin (Supplemental Table S1 and Supplemental Discussion).

Androgen Regulation of *Rhox* Genes

Spermatogenesis requires testosterone, the hormone that mediates its effects by binding to the androgen receptor (AR), a member of the nuclear hormone receptor family. Sertoli cells (but not germ cells) express AR and respond to testosterone, which implicates these cells as a primary mediator of testosterone action in spermatogenesis. While AR-regulated transcription factors have been identified in some androgen-responsive cells, including those in the prostate, surprisingly few have been identified in Sertoli cells (Lim et al., 1994; Rao et al., 2003). Thus, there is a need to identify transcription factor genes regulated by testosterone in Sertoli cells. To assess whether any of the *Rhox* genes are in this class, we used the androgen-responsive Sertoli cell line MSC1. We detected expression of five *Rhox* genes in MSC1 cells (*Rhox2*, *3*, *5*, *10*, and *11*). All five of these were also dramatically upregulated (5- to 7-fold) in response to incubation with testosterone and cotransfection with an AR expression plasmid. As a negative control, we used *Gata1*, which encodes a transcription factor expressed in an androgen-independent manner in Sertoli cells in vitro and in vivo (Yomogida et al.,

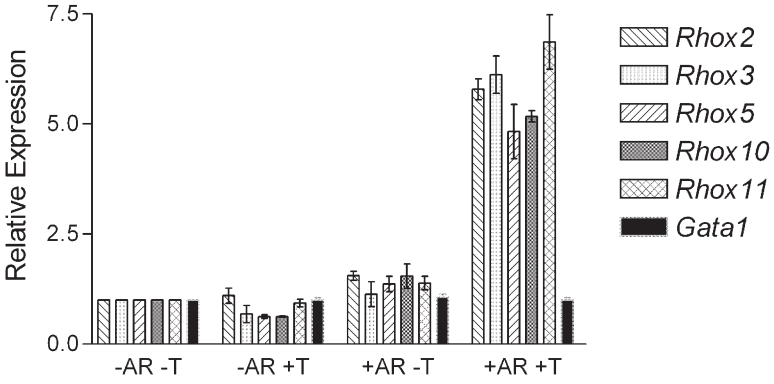

Figure 5. Androgen Regulation of *Rhox* Genes

MSC1 were transfected with an AR expression construct (1 μg) and cultured with 10 mM testosterone (T). Real-time RT-PCR showed that all *Rhox* genes expressed in MSC1 were positively regulated by androgen. The Sertoli cell-specific transcription factor, *Gata1*, was assayed as a nonandrogen regulated negative control.

1994). We found that *Gata1* mRNA levels remained unchanged in response to AR and testosterone in MSC1 cells (Figure 5), indicating that AR-induced expression of the *Rhox* genes was specific and not a global effect of AR stimulation. We conclude that at least 5 *Rhox* genes are androgen dependent and thus are candidates to regulate secondary androgen-responsive genes important for spermatogenesis.

Subfertility of *Rhox5* Null Mice

We assessed the reproductive function of one of the androgen-regulated *Rhox* genes, *Rhox5*, by closely examining the phenotype of *Rhox5* null mice, which previously had been shown to have no obvious defects (Pitman et al., 1998). Eight week timed matings showed these mice are subfertile. This analysis revealed that the *Rhox5* null mice produced on average only a single litter, in contrast to their wild-type (wt) littermates, which produced an average of 1.9 litters (Figure 6A). Litter number was reduced in pairings between null males with either wt or null females but not when wt males were mated with null females, indicating that the fertility defect was in the male. The fertility defect was even more obvious when mice pairs were allowed to mate for only 5 days, as 90% of wt pairs produced a litter, while only 30% of the null pairs did (n = 10 pairs each). Although *Rhox5* null male mice had an impaired ability to impregnate females, their litter sizes were normal (the average value was 4.9 and 5.0 for null and wt mice matings, respectively; n = 20 pairs each). The deficiency in male *Rhox5* null mice fertility was not due to alterations in sexual behavior, because null males generated a similar frequency of vaginal plugs (21/28 matings) to those generated by wt males during 3 day matings (24/28 matings; $p > 0.1$).

Defects in Spermatozoa Production and Motility in *Rhox5* Null Mice

We sought to determine why *Rhox5* null mice were hypofertile. We discovered that *Rhox5* null mice had ~2-fold less spermatozoa in the cauda epididymis than did wt littermates (Figure 6B). This difference was not due to loss of spermatozoa during transit through the epididymis, as there were also about half as many spermatozoa in the caput epididymis. Instead, a testis defect was responsible, based on several lines of evidence. First, the null mice had a modest but significant reduction in adult testes weight (Table 1). Second, null mice produce about half as many sonication-resistant spermatids (the most differentiated elongated spermatids) in the testis (Figure 6B). Third, in null mice there were fewer round and elongated spermatids per Sertoli cell in stage VII seminiferous-epithelial tubules (Figure 6C). This reduction indicated that the defect occurred in germ cells at the round-spermatid stage or earlier. Further evidence for an early, as opposed to a late, defect was that the null mice exhibited normal spermiation, based on quantitative analysis of stage VII and VIII tubules (data not shown).

We next addressed whether reduced germ cell output from the testis was due to increased germ cell death, decreased germ cell proliferation, or both. In normal testes, most apoptotic cells are either spermatogonia in stage I–IV tubules or meiotic spermatocytes in stage XII tubules (Hasegawa et al., 1997). In agreement, we found that wt testis sections had apoptotic cells only in these stages, as judged by TUNEL analysis (Figure 6E and Supplemental Figure S2). In *Rhox5* null mice, the number of TUNEL-positive cells in these stages increased dramatically (Figure 6D). In addition, the null mice had TUNEL-positive cells during stages VIII through XI that do not normally have apoptotic cells (Figure 6E). All or most of these apoptotic cells were spermatocytes, based on their position (most were in rows 2 to 4) and the presence of a meiotic spindle or decondensed chromatin in some. Although we cannot rule out that some spermatogonia also die during these stages as a result of loss of Rhox5, it is likely to be few, as less than one in ten of the TUNEL-positive cells resided directly adjacent to the basement membrane. The increased apoptosis was global, not in a few select tubules, as the number of TUNEL-positive cells *per positive tubule* was only modestly increased in *Rhox5* null animals (Figure 6D). The reduced germ cell output from *Rhox5* null testes appeared to be entirely due to increased germ cell apoptosis, as we did not observe a significant alteration in BrdU incorporation in *Rhox5* null testes (Table 1).

The effect of *Rhox5* loss was relatively specific, as we did not observe any other alterations in *Rhox5* null mice testes. They had normal seminiferous-tubule architecture and normal tubule diameter (Table 1). The number of Sertoli cells per tubule was similar between

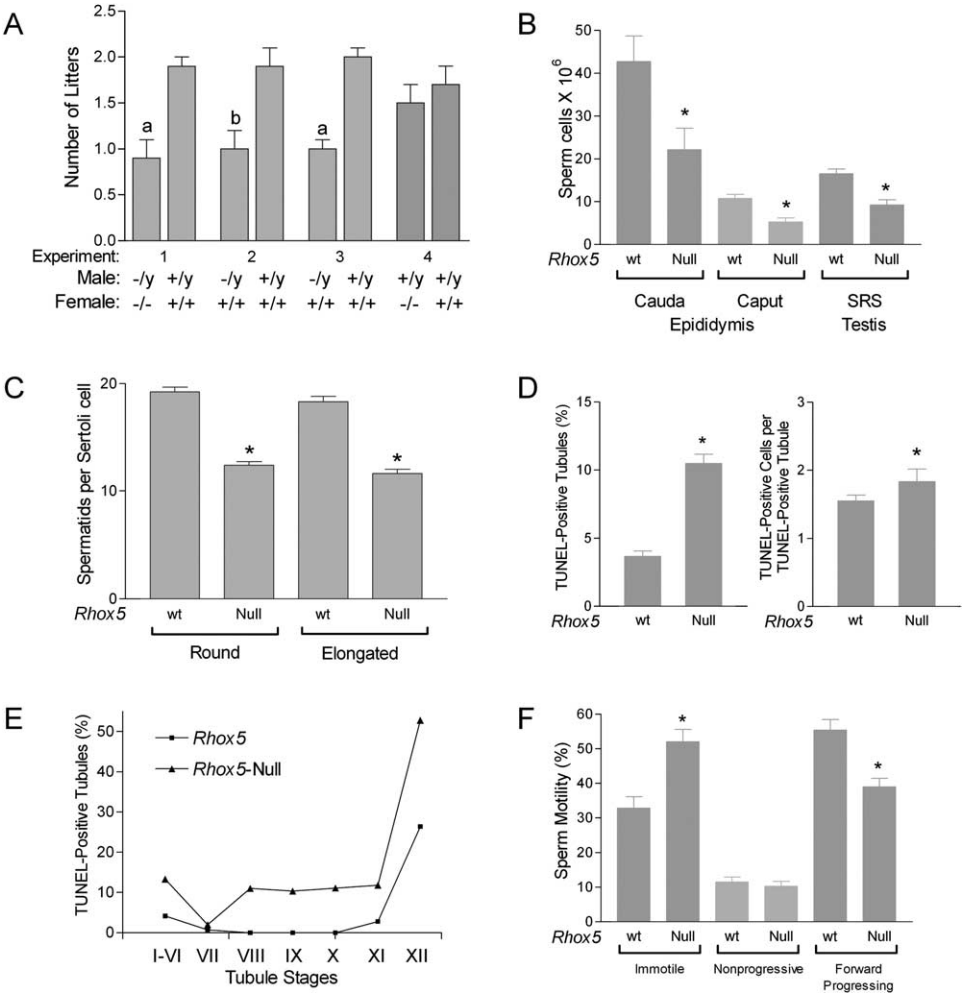

Figure 6. Subfertility of *Rhox5* Null Mice

(A) Eight week pairings of *Rhox5* null and control littermate mice. The mice were 6 weeks old at the beginning of cohabitation (except for experiment 3, in which eight-week-old mice were used). We used 20 pairs for each experiment (except for experiment 3, in which we used 10 pairs). Pairings containing *Rhox5* null male mice produced significantly fewer litters (Student's t test, a: $p < 0.001$; b: $p < 0.01$). Data represent the mean ± SEM.

(B) The number of caudal and caput epididymal sperm was counted in *Rhox5* null and control littermate (wt) mice. In both regions of the epididymis, *Rhox5* null animals had significantly fewer sperm than control mice (Student's t test, *$p < 0.001$). *Rhox5* null mice also had significantly fewer sonication-resistant spermatids (SRS) in their testes (Student's t test, *$p < 0.001$), based on analysis of four mice of each type.

(C) The testicular defect occurs prior to spermatid formation, as both round and elongated spermatids were reduced in number in stage VII tubules. Data represent the mean ± SEM for 12 mice in each group.

(D) *Rhox5* null mice had 3-fold more TUNEL-positive tubules than did wt mice (*$p < 0.001$). There was only a modest increase in the number of TUNEL-positive cells *per TUNEL-positive tubule*, indicating that loss of Rhox5 did not cause massive apoptosis in select tubules. The values shown are derived from examining three tissues sections from three null and three wt mice (n = 853 null tubule sections; n = 885 wt tubule sections).

(E) The percentage of TUNEL-positive tubules at different stages of the seminiferous epithelial cycle (determined from the tubules examined in [D]).

(F) Cauda sperm motility. There were significantly more immotile sperm and significantly less forward-progressing sperm in *Rhox5* null mice than in control littermate mice (Student's t test, *$p < 0.001$). Data represent the mean ± SEM for 10 mice in each group.

wt and *Rhox5* null animals as assessed by counting Sertoli cell nuclei located at the basement membrane in each tubule cross-section. Quantitative histological analysis showed that null testes contained the same proportions of tubules at each seminiferous epithelial stage as wt controls (Table 1; Dym and Fawcett, 1971). Null testes also had no changes in intratesticular testosterone levels (as assessed by ELISA assay, Table 1).

Because normal mice maintain some fertility even when their sperm counts are more drastically reduced than we observed for *Rhox5* null mice (Kumar et al., 2001b; Meistrich, 1993), we predicted that another factor must also contribute to the hypofertility of *Rhox5* null mice. In agreement with this prediction, we found that *Rhox5* null mice exhibited an increase in the percentage of immotile caudal spermatozoa compared to

Table 1. Characterization of the *Rhox5* Null Phenotype

	Null		wt	
Physical Parameters		n		n
Body weight (g)	24.8 ± 1.2	12	25.5 ± 1.8	12
Testis weight (mg)	88.7 ± 3.9 [a]	24	101.5 ± 2.1	24
Testosterone (ng/mg testis)	3.9 ± 1.0	6	4.2 ± 0.9	6
Fertility		n		n
Litter size	4.9 ± 0.5	20	5.0 ± 0.6	20
Sperm viability (%)	57 ± 1.8 [a]	6	67 ± 4.0	6
Abnormal sperm morphology (%)	6.3 ± 0.5	4	7.9 ± 1.1	4
Testis Cell Proliferation				
Total tubules	843	(%)	935	(%)
BrDu-positive tubles/section	24.8 ± 2.3	16.9	24.0 ± 1.7	17.1
BrDu-positive cells/tubule	2.0 ± 0.3		2.1 ± 0.1	
Histological Characterization		n		n
Tubule diameter (μm)	99.6 ± 1.0	6	98.2 ± 2.3	6
Total tubules	859	(%)	888	(%)
Spermatogenesis: Stage I–VI	57.8 ± 2.3	40.4	58.8 ± 3.7	39.7
Stage VII	25.1 ± 3.0	17.5	22.5 ± 2.0	15.2
Stage VIII	15.2 ± 1.5	10.6	14.7 ± 1.6	9.9
Stage IX	11.2 ± 0.6	7.8	13.8 ± 1.4	9.3
Stage X	4.5 ± 0.6	3.1	4.5 ± 0.9	3.0
Stage XI	9.8 ± 1.1	6.8	11.2 ± 1.0	7.6
Stage XII	13.8 ± 1.2	9.6	15.2 ± 0.9	10.2
Necrotic tubules per tissue section	3.2 ± 0.3	2.2	3.7 ± 0.6	2.5
Sertoli cells per tubule crossection	6.9 ± 0.4		6.8 ± 0.3	

Summary of physical and histological difference between wt and Rhox5 null animals (Student's t test, [a]$p < 0.001$). Data represent the mean ± SEM.

their wt littermates (Figure 6F). The percentage of forward-progressing spermatozoa was correspondingly reduced in the null mice. Thus, the hypofertility in *Rhox5* null mice may be the result of combined defects in both spermatozoa formation and maturation.

Discussion

We have identified a homeobox gene cluster on the X chromosome selectively expressed in reproductive tissues and placenta. The clustered arrangement of these *Rhox* homeobox genes stands in contrast to most homeobox genes, which are typically solitary genes dispersed throughout the genome (Duboule, 1994; Cillo et al., 2001). The only other mammalian homeobox clusters that have been identified are the large *Hox* gene clusters (Martinez and Amemiya, 2002) and smaller homeobox clusters (*NK*, *ParaHox*, and *Iroquois*) containing two to four genes (Brooke et al., 1998; Peterson, 2004; Pollard and Holland, 2000). We found that some members of the *Rhox* cluster are hormonally regulated and most are temporally and quantitatively expressed during testis development in a manner that corresponds to their position on the X chromosome. The cells responsible for expressing most of the *Rhox* genes in the testis are Sertoli cells. These cells are in direct contact with developing male germ cells and are essential for maintaining and directing spermatogenesis (Russell et al., 1993). We provide evidence that one of the *Rhox* genes expressed only in Sertoli cells within the testis is essential to promote the survival of germ cells and their proper maturation into motile sperm.

Why Is the *Rhox* Cluster on the X Chromosome?

The X chromosome linkage of the *Rhox* gene cluster is intriguing given the accumulating evidence suggesting that mouse and human X chromosomes harbor a higher proportion of genes involved in reproduction than do autosomes (Hurst, 2001). Recent genome-wide analyses indicate that genes preferentially expressed in some reproductive organs and cell types are overrepresented on the mouse X chromosome (Khil et al., 2004). Interestingly, these organs and cell types (placenta, ovary, and testicular somatic cells) are precisely the ones that express *Rhox* genes. While it is not known why sex-biased genes accumulate on the X chromosome, it has been proposed that it is due to sex-specific selective forces (Charlesworth, 2002; Vallender and Lahn, 2004). Genes expressed preferentially in males have been suggested to be overrepresented on the X chromosome because the single X chromosome present in males allows any recessive alleles that arise to be immediately acted upon by positive selection (Rice, 1994). Female-selective genes have been proposed to be on the X chromosome because this chromosome is present twice as often in females than males, allowing evolutionary forces twice the opportunity to select for genes benefiting females. The male-specific selective forces may also act on spermatogonia, as genes preferentially expressed in these early-stage male germ cells are also overrepresented on the X chromosome (Khil et al., 2004; Wang et al., 2001). In contrast, postmeiotic male germ cell-enriched genes are dramatically underrepresented on the X chromosome, presumably due to the inactivation of the X chromosome during meiosis (Khil et al., 2004).

While we do not know whether the X chromosomal localization of the *Rhox* gene cluster is critical for its function or confers some advantage during evolution, it is tempting to speculate that this location allowed the *Rhox* cluster to play a role in speciation. This hypothesis is consistent with several lines of evidence. First, X-linked genes are known to be largely responsible for the sterile males that arise from matings between species in which males are the heterogametic sex (Hurst, 2001). Second, the X-linked *Rhox* gene cluster seems a particularly good candidate to play a role in this hybrid sterility because most of the *Rhox* genes are expressed in the male reproductive tract and all of them encode putative transcription factors that have the potential to regulate many other genes. Third, at least one member of the *Rhox* gene cluster, *Rhox5*, has another key feature required to drive speciation: it has undergone rapid evolution due to positive selection (Maiti et al., 1996; Sutton and Wilkinson, 1997a). This rapid divergence contrasts with the strong conservation of many transcription factors, including Hox transcription factors (Duboule, 1994), but it is typical of genes involved in reproduction, including the male-determining transcription factor SRY (Swanson and Vacquier, 2002). Lastly, there are several parallels between *Rhox5* and a *Drosophila* gene that has been recently shown to be responsible for hybrid sterility in flies: *odyssus* (*OdsH*). Like *Rhox5*, *OdsH* is a homeobox gene undergoing rapid evolution that is normally expressed in the male reproductive tract (Sun et al., 2004). Like *Rhox5*, *dsH* is not absolutely required for spermatogenesis, but rather its loss causes modest reproductive defects, including subfertility (Sun et al., 2004).

Rhox5 Null Mice Suffer from Male Reproductive Defects

We found that a null mutation in *Rhox5* results in reproductive dysfunction (Figure 6 and Table 1). Male *Rhox5* null mice suffered from hypofertility and had reduced numbers of round and elongated spermatids in the testis. This depletion was due, at least in part, to increased apoptosis of meiotic germ cells in the testis. Apoptosis is a normal process associated with spermatogenesis that is enhanced by gonadotropin withdrawl, heat stress, torsion, and assault by many toxic biochemical agents (Hikim et al., 2003; Lysiak et al., 2001). Interestingly, *Rhox5* null testes exhibited an increased frequency of apoptosis in both germ cells that normally die (stage I to IV spermatogonia and stage XII spermatocytes) as well as those that do not normally die (stage VII to XI spermatocytes). This apoptosis could be due to loss of a Rhox5-dependent survival factor presented by Sertoli cells to germ cells. In agreement with this possibility, a microarray analysis we have performed revealed that loss of *Rhox5* in the testis alters the expression of several genes encoding secreted proteins that regulate fatty acid and sugar metabolism (J.A.M. and M.F.W., unpublished observations). Alternatively, Rhox5 may normally function as a checkpoint surveillance transcription factor that, if absent, causes premature entry to the next stage and therefore an increased sensitivity to apoptosis. Loss of androgen signaling within the testis results in increased apoptosis beginning in mid stage VII and continuing through stage IX (Lue et al., 2000). Because *Rhox5* is regulated by androgen, it is tempting to speculate that Rhox5 is one mediator of apoptotic survival lost when androgen is deprived. However, the relatively modest increase in stage VII apoptosis (2- to 3-fold) in *Rhox5* null animals suggests that other factors may also be involved and/or that other *Rhox* genes may compensate at stage VII.

In addition to reduced germ cell output, *Rhox*5 null mice had fewer than the normal proportion of motile sperm. This motility defect may be due to improper germ cell maturation in the testis as a result of loss of *Rhox5* in the adjacent Sertoli cells. Alternatively, it could be due to improper sperm maturation in the caput, the region of the epididymis where sperm normally acquire forward motility, and the only region of the epididymis that expresses *Rhox5* (Rao et al., 2002b). It should be noted that while the epididymal and testicular phenotypic effects resulting from loss of *Rhox5* were reproducible and statistically significant, they were not dramatic. This may be due to compensatory effects of one or more of the other *Rhox* genes expressed in the epididymis and testis. Consistent with this possibility, loss of *Rhox5* increases the testicular expression of two genes in the upstream subcluster: *Rhox2* and *Rhox3* (J.A.M. and M.F.W., unpublished data).

Rhox Genes Are Expressed during Specific Phases of Spermatogenesis

While many *Rhox* genes may mediate redundant functions, the nonoverlapping expression patterns of some *Rhox* genes suggest they perform distinct functions. The first gene in the *Rhox* cluster, *Rhox1*, is expressed and shut off at key time points during the first wave of spermatogenesis. *Rhox1* is expressed in Sertoli cells when they are actively dividing; it shuts off when these cells cease to divide and undergo terminal differentiation (between days 10 and 15 postpartum). This striking correlation suggests the possibility that Rhox1 promotes Sertoli cell proliferation and prevents Sertoli cells from undergoing premature terminal differentiation. Rhox1 may also regulate germ cell events, since spermatogonia are undergoing proliferation when Rhox1 is expressed in the neighboring Sertoli cells. When Rhox1 expression wanes, most of the spermatogonia differentiate into nondividing meiotic spermatocytes. This suggests a model in which Rhox1 regulates the transcription of Sertoli cell genes that encode cell-surface or secreted proteins that interact with germ cells to promote their proliferation and/or inhibit their differentiation. Rhox1 may also inhibit the premature differentiation of cells in the ovary, since *Rhox1* is the only *Rhox* gene highly expressed in the ovary, an organ that is maintained in an inactive state until germ cells in specific follicles are recruited during ovulation. Immature granulosa cells rapidly lose *Rhox1* expression when folliculogenesis is induced in naive ovaries in response to LH and FSH analogs in vivo (J.A.M. and M.F.W., unpublished data).

We speculate that the function of other *Rhox* genes also corresponds to when they are expressed. For example, the timing of *Rhox10* expression suggests *Rhox10* may regulate the proliferation of spermatogo-

nia or their differentiation into meiotic-competent spermatocytes (Figure 4D). The peak expression of *Rhox2*, *5*, *8*, and *10* correlates with the end of Sertoli-cell proliferation and the transition of preleptotene to diplotene spermatocytes. *Rhox7* expression correlates with the progression of secondary spermatocyte development. Finally, the timing of *Rhox3* and *11* suggests that they may regulate the expression of factors that govern spermatid maturation. While these proposed functions are based solely on simple correlations between *Rhox* gene expression patterns and known events during the first wave of spermatogenesis, we suggest they provide a useful framework for future investigations.

Rhox proteins have novel sequence features that suggest that they bind to a different class of gene promoters than do previously studied homeobox proteins. Most Rhox proteins possess unusual amino acids at the four positions in the homeodomain known to make base-specific contacts with DNA. For example, while position 51 in virtually all previously defined homeobox proteins is an N that binds to an adenine (Duboule, 1994), only one of the eleven Rhox homeodomains has an N at this position (Figure 2A). While some Rhox proteins have a polar-uncharged amino acid related to N (M or S), most have a positively charged amino acid (K or R) or a hydrophobic amino acid (I or Y). Likewise, most Rhox proteins have an atypical amino acid at position 50, which has been shown in previous studies to be critical for dictating DNA binding specificity (Treisman et al., 1989). While this position is an E in virtually all Hox and Hox-related genes (Duboule, 1994), most Rhox proteins have a K at this position (Figure 2A). The common occurrence of K50 coupled with the similarity between Rhox and Paired-like homeodomains at other positions (Figure 2B) suggests that the *Rhox* genes may have evolved as an offshoot of the K50 subclass of the Paired-like genes (Galliot et al., 1999). Position 47 also contains unusual amino acids; instead of the hydrophobic amino acids found in most previously studied homeobox proteins (typically I or V), most Rhox proteins have polar-charged amino acids (D, E, or R) or N. Paradoxically, the least conserved base-specific contact residue in previously studied homeobox proteins, position 54 (Duboule, 1994), is the most conserved position in Rhox homeodomains, although some variation occurs even at this position (Figure 2A). The net result of the amino-acid heterogeneity at these four positions (47, 50, 51, and 54) is that each of the 12 Rhox proteins has a unique set of amino acids at the positions predicted to make base-specific contacts with DNA (Figure 2A). It remains to be determined whether this reflects distinct DNA binding specificities, different strategies for binding to DNA, or both.

Rhox Cluster Evolution

In contrast to the ancient *Hox* gene clusters, which have evolved to direct conserved body plan and limb developmental pathways maintained in many animal species from insect to man, the *Rhox* cluster appears to have evolved more recently. *D. melanogaster* has only a single gene related to *Rhox* genes: aristaless (*al*); it contains two introns at the signature positions characteristic of *Rhox* genes (Duboule, 1994), and its homeodomain is as related with Rhox homeodomains as are most Rhox homeodomains with each other (J.A.M. and M.F.W., unpublished data). A large rat *Rhox* gene cluster exists (J.A.M., S.R.B., and M.F.W., unpublished data), but extensive searching has revealed only two human *RHOX* orthologs (Geserick et al., 2002; Wayne et al., 2002).

The existence of a large gene cluster devoted to reproduction in rodents but not in humans is consistent with the greater reproductive capacity of rodents. Precedence for this pattern of gene evolution comes from the prolactin and vomeronasal receptor clustered gene families, both of which are involved in reproduction and have many more members in rodents than in humans (Lane et al., 2002; Rodriguez et al., 2002; Soares and Linzer, 2001). Another striking example of this phenomenon is the massive rodent-specific expansion of the olfactory receptor genes. This along with the selective loss and inactivation of olfactory receptor genes in mammals probably significantly contributed to the greater olfactory capabilities of rodents as compared with humans (Gilad et al., 2003; Reed, 2004).

Why might rodents need a greater number of Rhox proteins than would primates? One possibility lies in the different ways spermatogenic waves are organized between primates and rodents. A cross-section of a rodent testis tubule contains Sertoli cell-germ cell associations from only a single stage of the seminiferous epithelial cycle (there are 12 stages in the mouse and 14 stages in the rat), whereas a cross-section from a primate testis tubule contains cell-cell associations indicative of multiple stages occurring simultaneously (Clermont, 1963). Thus, only in rodent seminiferous tubules does the stage vary in a discrete manner along the length of the tubule (e.g., stage I followed by stage II, etc.). This dramatic difference between rodent and primate spermatogenesis leads us to speculate that the *Rhox* genes play a role in this difference. Perhaps the rigid stage-to-stage boundaries are established by *Rhox* genes in a spatial colinear fashion, similar to how *Hox* genes establish body segmentation. Such an arrangement would necessitate more *Rhox* genes in rodents than in primates. A second explanation for why humans have few RHOX proteins is that it is compensated for by an expansion of human genes encoding cofactors that cooperate with RHOX proteins. One set of candidates to fulfill this role is the SPANX genes, which have at least six members in humans and apes, but have only a single ortholog in rodents (Kouprina et al., 2004). SPANX genes are expressed specifically in testis and encode nuclear proteins. Interestingly, the N-terminal domains of the SPANX proteins share some sequence identity with the N-terminal domains of the Rhox/RHOX proteins (J.A.M. and M.F.W., unpublished data), suggesting that these as yet uncharacterized protein motifs may serve similar functions.

Colinearity

Why are *Rhox* gene family members clustered? One possibility is that *Rhox* genes have arisen as a result of recent duplication events and thus have not yet had an opportunity to disperse to other chromosomal locations. Another possibility is that *Rhox* genes have

somehow become dependent on one another over evolutionary time and thus selection pressure has maintained their intimate relationship at a single chromosomal site. The latter possibility seems to be the case for the *Hox* gene clusters, as they are ancient gene arrays regulated by transcriptional enhancers shared by several individual *Hox* genes (Duboule, 1994; Gould et al., 1997; Spitz et al., 2003). While discrete transcriptional enhancer elements have yet to be precisely defined, enhancer activities have been identified at chromosomal regions in the termini of some *Hox* subclusters. Evidence suggests that these terminal chromosomal regions dictate the colinear expression pattern characteristic of *Hox* gene clusters (Duboule, 1998; Spitz et al., 2003).

In the case of the *Rhox* gene subclusters, we have no direct evidence that enhancer elements dictate their colinear pattern of expression. However, our discovery that two of the *Rhox* subclusters exhibit temporal colinearity and all three *Rhox* subclusters exhibit quantitative colinearity (Figure 4) is consistent with the existence of titratable global enhancer elements at one end of each of the three *Rhox* subclusters. A long-distance global enhancer that acts over the entire length of the *Rhox* cluster (~0.7 Mb) may also exist, as the most 5′ *Rhox* gene (*Rhox1*) is the first *Rhox* gene to be expressed postnatally in the testis and the most 3′ *Rhox* genes (*Rhox11* and *12*) are expressed last. We note, however, that a simple model involving only global enhancers does not fully explain *Rhox* gene regulation, just as it does not fully explain *Hox* gene regulation (Gould et al., 1997). Independent regulatory control must have evolved for the subcluster β gene *Rhox5*, as we previously showed that only 0.6 kb of 5′ flanking sequence upstream from its proximal promoter is sufficient to drive its normal developmental expression pattern in a cell type-specific manner in the testis and epididymis in vivo (Rao et al., 2002b, 2003). In addition, *Rhox5* has a second (distal) promoter selectively expressed in placenta and ovary that is independently regulated by different transcription factors than those that regulate the proximal promoter (Rao et al., 2002a). A similar arrangement of male- and female-specific promoters may drive the transcription of the other *Rhox* genes that are expressed in both male and female reproductive tissues. Conversely, *Rhox* genes expressed predominantly in only male or female reproductive tissues, not both, may possess only a single promoter. For example, the subcluster β gene, *Rhox6*, which is expressed in placenta but hardly, if at all, in testes (at least 1000 times less; Figure 3), may lack a male-specific promoter.

We do not know whether *Rhox* colinearity extends beyond the example we described here. Most of the *Rhox* genes are expressed in the adult testis, which contains seminiferous tubules at all stages of spermatogenesis (Figure 4). It will be intriguing to know whether the *Rhox* genes are expressed in a stage-specific manner during adult spermatogenesis that corresponds to their expression pattern. The *Rhox* gene cluster may also exhibit a colinear expression pattern in the female reproductive tract, either during the ovarian cycle or as placental development proceeds. The discovery of the *Rhox* homeobox gene cluster provides a unique opportunity to study the evolution, regulation, and function of a set of related transcription factors likely to be devoted to regulating reproduction.

Experimental Procedures

Animals

All experiments were performed in accordance with National Institutes of Health guidelines for care and use of animals. The *Rhox5* null mice are described in Pitman et al. (1998). For tissue preparation and sperm counting, mice were sacrificed by cervical dislocation and then analyzed as described previously (Zhao et al., 2001). Testosterone was measured using the ACTIVE Testosterone ELISA assay (Diagnostic Systems Laboratories, Webster, Texas).

Cell Culture and Transient Transfection Assays

The MSC1 Sertoli cell line was provided by Dr. Michael Griswold (Washington State University, Pullman, Washington) and cultured in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum and 50 μg/ml penicillin and streptomycin. Cells were transiently transfected with an AR expression plasmid for two days as previously described (Rao et al., 2003).

Identification of the *Rhox* Genes

Pem, *Psx1*, and *Psx2* homeodomain sequences were used as probes to BLAST search regions of the mouse X chromosome. The predicted exon and intron sequences were mapped and compared to the structure of the *Pem* gene to determine whether the novel homeobox sequences we identified were likely to belong to the same subfamily. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar et al., 2001a).

The most 5′ in-frame ATG in each *Rhox* cDNA was designated as the start codon. While we could not be certain that this ATG was the start codon in all cases, three lines of evidence suggest that it was: first, the start ATGs that we identified for all 12 *Rhox* genes conformed to the Kozak consensus sequence at the two critical positions; all had a purine upstream at the –3 position and all had a G downstream at the +4 position. The Pedersen algorithm, which gives scores above 0.5 for bona fide start ATGs, yielded scores between 0.625 and 0.8 for the ATGs we selected (Pedersen and Nielsen, 1997). Second, the amino acid following the ATG that we selected was the same (Glu) for all Rhox genes except for *Rhox3*, *11*, and *12*. Third, Rhox4, 6, 7, 8, 9, 11, and 12 had in-frame stop codons upstream of the start ATG, thereby ruling out the existence of another start ATG upstream.

Gene Expression Analysis

For real-time reverse transcriptase (RT)-polymerase chain reaction (PCR) analysis, cDNAs were generated by using the *iScript* RT kit (BioRad laboratories, Hecules, California). We chose primers that were highly specific for each of the 12 genes and that spanned one intron to eliminate the possibility of genomic contamination contributing to the Ct signal. Real-time production of *Rhox* gene products was measured by SYBR green fluorescence and then compared by the delta Ct method. Expression data are presented as relative expression above background signal and normalized to the L19 housekeeping transcript. Similar results were obtained with β-actin. The data presented are, at a minimum, the average value of two separate tissue RT reactions assayed in duplicate. Following real-time data acquisition, melt curve analysis was used to ensure that Ct values were not inflated by the production of primer dimers or other aberrant PCR products.

Supplemental Data

Supplemental Data include two figures and a table and can be found with this article online at http://www.cell.com/cgi/content/full/120/3/369/DC1/.

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